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(71)(72) Applicant and Inventor: ZILTENER, Hermann, J. [CA/CA]; 3649 West 19th Avenue, Vancouver, Britisch Columbia V6S 1C5 (CA).

(74) Agents: NASSIF, Omar, A. et al.; McCarthy Tetrault, Suite 4700, Toronto Dominion Bank Tower, Toronto-Dominion Centre, Toronto, Ontario M5K 1E6 (CA).

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(54) Title: METHOD FOR THE DETECTION OF PHOSPHOTYROSINE RESIDUES

(57) Abstract

There is disclosed a sensitive and rapid method for detecting phosphotyrosine residues using particle concentration fluorescence immunoassay. There is further disclosed methods for measuring the activity of protein-tyrosine kinases and phosphatases, for screening substrates for protein-tyrosine kinases and phosphatases, and for screening agents for their ability to modulate the activity of protein-tyrosine kinases and phosphatases or modulate the specificity of a substrate thereto.

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Method for the detection of phosphotyrosine residues

DESCRIPTION

Technical Field

The present invention relates generally to a method for detecting phosphotyrosine residues and, more specifically, to a method for detecting protein-tyrosine kinases and protein-tyrosine phosphatases.

Background of the Invention

The control of cellular activation, growth, and differentiation by mitogens and other cytokines is often achieved via alterations in the tyrosine phosphorylation states of regulatory proteins (Pelech et al., Biochem. Cell. Biol. 68:1297-1330, 1990). Several mitogens, such as epidermal growth factor, macrophage colony stimulating factor-I, and the steel locus product, all bind to cell surface receptors that are protein-tyrosine enzymes specified by proto-oncogenes (Ullrich et al., Nature 309:418-425, 1984; Hampe et al., Proc. Natl. Acad. Sci. USA 81:85-89, 1984; Huang et al., Cell 63:225-233, 1990). However, for the vast majority of proto-oncogene-encoded receptor-like protein-tyrosine kinases, the ligands remain to be identified. Activation of the oncogenic potential of these and non-receptor protein-tyrosine kinases such as $p60^{src}$ (Takeya et al., J. Virol. 44:12-18, 1982) and $p56^{lck}$ (Marth et al., Cell 43:393-404, 1985) results from mutations which lead to a high level of constitutive phosphorylating activity.

Protein-tyrosine phosphatases are being discovered at an increasing rate, largely by recombinant techniques on the basis of a phosphatase consensus sequence (Jirik et al., FEBS Lett. 273:239-242, 1990). For example, recent studies have demonstrated the occurrence of complementary receptor-like protein-tyrosine

phosphatases, such as CD45 (Tonks et al., Biochemistry 24:8695-8701, 1988), LAR (Streuli et al., J. Exp. Med. 5:1523-1530, 1988), and LRP (Matthews et al., Proc. Natl. Acad. Sci. USA 12:4444-4448, 1990), for which the interacting surface molecules are still not known.

The rate of identification of new protein-tyrosine kinases and phosphatases has greatly overtaken their enzymological characterization. This reflects, in part, the low levels of expression of these regulating enzymes which necessitates highly sensitive assays. Standard radioisotope filter-type assays that are currently employed are slow and lack the sensitivity required to confidently identify phosphotyrosine residues. In addition, such assays, due to their utilization of radioisotopes, require special handling and disposal of both the reagents and waste products.

Accordingly, there is a need in the art for a fast, convenient and highly sensitive assay which detects the presence of phosphotyrosine residues, and may be used to test for the activity of protein-tyrosine kinases and phosphatases.

Summary of the Invention

It is an object of the present invention to provide a highly sensitive and rapid method for detecting phosphotyrosine residues without the use of radioisotopes. Upon further study of the specification and appended claims, additional objects and advantages of this invention will become apparent to those skilled in the art.

The present invention provides a sensitive and rapid method for the detection of phosphotyrosine residues by an immunological assay technique, and preferably by particle concentration fluorescence immunoassay (PCFIA). In various embodiments, the assay of the instant invention is useful in the screening of large numbers of samples for the presence of protein-tyrosine kinases and phosphatases,

in the screening of large number of samples of substrates for protein-tyrosine kinases and phosphatases, and in screening for substances that can modulate protein-tyrosine kinase and phosphatase activity either by inhibition or activation, or by modulating substrate specificity.

More specifically, in one aspect, the present invention is directed to a method for detecting a phosphotyrosine residue by contacting the phosphotyrosine residue with an anti-phosphotyrosine antibody which binds to the phosphotyrosine residue, and detecting the anti-phosphotyrosine antibody by an immunological assay technique, and preferably by particle concentration fluorescence immunoassay.

In another aspect, the present invention discloses a method for measuring the activity of a protein-tyrosine kinase or phosphates within a sample by immobilizing a substrate on a solid support. The substrate containing a tyrosine or phosphotyrosine residue; exposing the substrate to the sample such that the protein tyrosine kinase or phosphatase within the sample phosphoralates the tyrosine or dephosphorolates the phosphotyrosine residue of the substrate to form a phosphotyrosine or tyrosine residue, respectively; contacting the substrate with an anti-phosphotyrosine antibody which binds to the phosphotyrosine residue; and detecting the anti-phosphotyrosine antibody by an immunological assay technique, and preferably by particle concentration fluorescence immunoassay.

In still another aspect, the present invention is directed to a method for screening for substrates for protein-tyrosine kinases or phosphatases by immobilizing the substrate on a solid support; exposing the substrate to the protein-tyrosine kinase or phosphatase; contacting the substrate with an anti-phosphotyrosine antibody; and detecting the anti-phosphotyrosine antibody by an

immunological assay technique, and preferably by particle concentration fluorescence immunoassay.

In yet another aspect, the present invention discloses a method for screening an agent for its ability to modulate the activity of a protein-tyrosine kinase or phosphatase, or modulate the specificity of a substrate to the protein-tyrosine kinase or phosphatase, by immobilizing the substrate on a solid support, the substrate containing a tyrosine or phosphotyrosine residue; exposing the substrate to the protein-tyrosine kinase or phosphatase in the presence of the agent; contacting the substrate with an anti-phosphotyrosine antibody; and detecting the anti-phosphotyrosine antibody by an immunological assay technique, and preferably by particle concentration fluorescence immunoassay.

In each of the above aspects, the anti-phosphotyrosine antibody may be fluorescently labelled and directly detected by PCFIA. Alternatively, the antiphosphotyrosine antibody may be further contacted with a fluorescently labelled antibody which binds to the anti-phosphotyrosine antibody and indirectly detected by PCFIA.

In an alternative aspect, the immunological technique for detecting the anti-phosphotyrosine antibody is an Enzyme-Linked Immunosorbent Assay (ELISA).

These and other aspects will become evident upon reference to the following detailed description and attached drawings.

Brief Descriptions of the Drawings

Figure 1(a) illustrates serial dilutions of partially purified $p56^{lck}$ preparations tested for protein phosphotyrosine activity using PCFIA and MAb PY20 (open symbols) and [^{32}P]-incorporation filter assay (closed symbols). Parameters are optimized for PCFIA (open and closed squares) and optimized for the filter assay (open and closed circles). Figure 1(b) illustrates $p56^{lck}$ catalyzed phosphotyrosine residues measured using the

anti-phosphotyrosine MAb PY20 (open squares) or MAb 4G10 (open circles) under optimized PCFIA conditions.

Figure 2 illustrates serial dilutions of $p56^{lck}$ tested for protein-tyrosine kinase activity using: (1) the substrate MPB (solid line) coupled to amino-activated microsphere particles via carboxy residues (open squares) or coupled to carboxy-activated microsphere particles via amino residues (open circles), and (2) the synthetic peptide corresponding to the $p56^{lck}$ autophosphorylation site (Tyr 394) (dotted line) coupled to amino-activated microsphere particles via carboxy residues (open squares) or coupled to carboxy-activated microsphere particles via amino residues (open circles).

Figure 3 illustrates protein-tyrosine kinase activity of column fractions obtained after ion exchange chromotography of cell extracts obtained from LSTRA cells, Figures 3(a) and 3(b), or YAC cells, Figure 3(c). In Figure 3(a), a 1/5 dilution of fractions obtained from 10^9 LSTRA cells were tested using the [^{32}P] filter assay. In Figure 3(b), a 1/150 dilution of the above fractions from LSTRA were tested using a method of the present invention. In Figure 3(c), a 1/5 dilution of fractions obtained from 10^8 YAC cells were tested using a method of the present invention.

Figure 4(a) illustrates serial dilutions of alkaline phosphatases (open circles) and potato acid phosphatase (open squares) tested for protein-tyrosine phosphatase activity using tyrosine phosphorylated MBP as the substrate. Figure 4(b) illustrates the inhibition of potato acid phosphatase activity with serial dilutions of vanadate (open circles) and molybdate (open squares) added to filtration plate wells containing 1 U/ml of potato acid phophatase and phosphorylated MBP substrate.

Detailed Discussion of the Invention

The present invention discloses a sensitive and rapid assay for the detection of phosphotyrosine residues

by an immunological assay technique, and preferably by particle concentration fluorescence immunoassay (PCFIA). This particular assay is fast, highly sensitive and does not employ radioactive isotope labels. Furthermore, this assay permits the screening of large numbers of samples for the presence of protein-tyrosine kinases or phosphatases, as well as the screening of large numbers of samples for substrates specific to these enzymes. PCFIA also allows for the screening of substances which can modulate (i.e., inhibit or activate) protein-tyrosine kinase and/or phosphatase activity, or can modulate substrate specificity.

The substrates of the present invention include proteins and synthetic peptides which contain tyrosine or phosphotyrosine residues. Tyrosine and phosphotyrosine residues are represented by the formulas X-CH_2-benzyl-OH and X-CH_2-benzyl-phosphate (i.e., para-substituted), respectively, wherein X is the protein or synthetic peptide. For example, the myelin-basic protein and a synthetic peptide corresponding to the autophosphorylation site of $p56^{lck}$ perform equally well in the detection of $p56^{lck}$ kinase activity obtained from a recombinant source. Similarly, the myelin-basic protein phosphorylated on tyrosine residues by $p56^{lck}$ may be employed as the substrate in the detection of phosphatase activity.

The solid support to which the substrate is immobilized may be any of a variety of materials commonly employed. In the case of PCFIA, Fluoricon™ (Idexx, Portland, Maine), carboxyl activated polystyrene particles are preferred.

Following immobilization on the solid support, the substrate is exposed to either a kinase or phosphatase in the presence of a suitable medium. Suitable mediums include ATP in combination with a buffer. When the substrate contains tyrosine residues, exposure to a kinase results in the phosphorylation of the tyrosine residues to phosphotyrosine. When the substrate includes

phosphotyrosine residues, exposure to a phosphatase results in the dephosphorylation of the phosphotyrosine residue. The increase or decrease of phosphotyrosine residues may then be detected, such as by PCFIA.

Detection of the phosphotyrosine residues is accomplished by exposing the phosphotyrosine residues to an anti-phosphotyrosine antibody. Such an antibody may be either monoclonal ("MAb") or polyclonal, and is selected such that it binds to the phosphotyrosine residues. Monoclonal antibodies specific for phosphotyrosine residues may be produced in the following manner. Mice are immunized with phosphotyrosine that has been chemically crosslinked using EDC to KLH (keyhole limpet hemocyanine). After repeated immunizations the spleen cells of the immunized mouse are fused with myeloma cells (e.g., X 631A98.653) according to general protocols (J.W. Goding, "Monoclonal Antibodies Principles and Practise," Golding, J.W., ed., Acad. Press, N.Y., 1986). Hybridoma supernatants are then screened for presence of monoclonal antibodies that are specific for phosphotyrosine residues and do not crossreact with phosphoserine or phosphothreonine residues using the PCFIA technique. The screening is performed by incubating aliquots of hybridoma supernatant in the filtration plate with beads that are coated with BSA that have been chemically crosslinked (using EDC) with either phosphoserine, phosphothreonine or phosphotyrosine residues. Antibody bound to any of the beads will be detected by the addition of a fluorescinated second antibody that is specific for mouse immunoglobulin. Hybridomas that secrete antibodies that bind to phosphotyrosine but not to phosphoserine or phosphothreonine may then be subcloned and cultivated for monoclonal antibody production.

The antibody may be labelled directly with a fluorescent marker detectable by PCFIA, or may be labelled indirectly by contact with a second antibody labelled with a fluorescent marker. This second antibody is chosen such

that it binds to the anti-phosphotyrosine antibody and is detectable by PCFIA.

When compared to a standard [^{32}P] filter-type assay, the preferred assay of the present invention achieves a 100-fold higher sensitivity. While the dose response curve for the instant method is non-linear, the calculation of absolute amounts of phosphotyrosine residues can be obtained from standard curves run in parallel in the same assay. The PCFIA method of the present invention is specific for phosphotyrosine kinases/phosphatases, and no signal from protein-serine/threonine kinases is measured. Thus, the PCFIA is more specific than filter assays, particularly when protein substrates like MBP, casein, etc., are used and only partially purified protein-tyrosine kinase is being tested.

The following experimental examples are offered by way of illustration and should not be construed as limitations to the present invention.

EXAMPLES

Reagents and Buffers:

Bovine brain myelin basic protein (MBP), 2-[N-morpholino] ethanesulfonic acid (MES), sodium orthovanadate (Na_3VO_4), sodium molybdate (Na_2MO_4), polyglutamic acid tyrosine (4:1) and trisma base (Tris) were purchased from Sigma (St. Louis, Missouri). ATP, bovine serum albumin (BSA), 3-[N-morpholino], propane sulphonic acid (MOPS), dithiothreitol (DTT), phenylmethylsulphonylfluoride (PMSF) potato acid phosphatase (PAP, 60 U/ml), and alkaline phosphatase (AP, 100 U/ml) were obtained from Boehringer Mannheim (Laval, Quebec). Ethylenediaminetetraacetic acid (EDTA), nonidet P-40 (NP40) , NaN_3, NaCl, $MNCl_2$, glycerol, and phosphoric acid were purchased from BDG (Toronto, Ontario). Newborn

bovine serum (NBS) and 10x phosphate buffered saline (PBS) were from Gibco (Grand Island, New York). [1,4-piperazine bis (ethanesulfonic acid)] (PIPES) was from Aldrich (Milwaukee, Wisconsin). (1-ethyl-3-3(3-dimethyl amino propyl) carbodiimide-HCL) (EDC) was from Pierce (Rockford, Illinois). [γ-^{32}p] ATP, (4500 Ci/mMole, 10 mCi/ml), and Ecolume scintillation fluid were from ICN (Mississauga, Ontario). Polyclonal goat anti-mouse FITC conjugated antibodies were obtained from Calbiochem (San Diego, California). Antiphosphotyrosine monoclonal antibodies were purchased from ICN (Mississauga, Ontario) (PY20) (10), Upstate Biotechnology, Inc. (Lake Placid, New York (4G10) (11) or Boehringer Mannheim (Laval, Quebec) (1G2).

The $p56^{lck}$ used in these experiments is a DEAE-Sepharose fraction of either sf9 cell lysates over expressing recombinant $p56^{lck}$ using a baculovirus expression system, or LSTRA cell membranes, a mouse cell line known to over express $p56^{lck}$ (Marth et al., Cell 43:393-404, 1985). The enzyme eluted in 25 mM Tris HCl, pH 7.5, 0.1 M glycerol, 1 mM EDTA, 1mM PMSF, 0.1% NP40 at about 350 mM NaCl.

Buffers are as follows:

Buffer A:	20 mM Tris-HCl (pH 7.5), 0.2 mM DTT, 0.5% BSA, 4 mM Na_3VO_4
Buffer B:	1xPBS (pH 7.3), 2% NBS (0.2 μm filtered), 0.2% w/v NaN_3
Buffer C:	20 mM MOPS (pH 7.5), 0.2 mM DTT, 10 mM $MnCl_2$, 0.1 Mm Na_3VO_4, 100 mM NaCl, 5% glycerol
Buffer D:	20 mM PIPES (pH 6.0), 0.5% BSA
Buffer E:	20 mM Tris (pH 9.6), 0.5% BSA

Instrumentation:

Protein-tyrosine kinase and phosphatase assays are performed by particle concentration fluorescence immunoassay (PCFIA) using a Screen Machine manufactured by Idexx (Portland, Maine). The assays are performed in filtration plates that have a standard 96-well microtitre format. The wells are conical and have a 0.2 μm filter at their base. Below the filter is a sump to which vacuum can be applied. Substrate proteins or peptides are immobilized on 0.8 μm diameter polystyrene particles which are added to the plates. Prepared plates are then applied to the machine, where unbound reagents are washed through the filter and drained to waste. Washing cycles and the addition of reagents such as antibodies labelled with fluorochrome are automatically performed on all 96 samples in parallel. Fluorescent intensity is measured in each well. Up to ten 96-well plates can be assayed in one run using this system.

EXAMPLE 1

Substrate Immobilization to Assay Particles

Fluoricon™ 0.8 μm diameter carboxyl activated polystyrene particles (Idexx, Portland, Maine) are coupled with myelin basic protein (MPB) or a peptide (lck peptide) derived from $p56^{lck}$ autophosphorylation containing the sequence (SEQ ID NO: 1):

Lys Lys Gly Gly Arg Leu Ile Glu Asp Glu
Tyr Thr Ala Arg Gln Gly Gly Lys Arg Leu
Ile Glu Asp Glu Tyr Thr Ala Arg Gln

The peptide contains an imperfect tandem repeat of the site of the $p56^{lck}$ autophosphorylation site (Bolen et al., Trends Biochem Sci. 14:404-407, 1989) (residue 394) and two terminal lysine residues for coupling via NH3 groups in a carbodiimide (EDC) mediated coupling reaction. The peptide is synthesized using an Applied Biosystems Model 430A peptide synthesizer purified by reverse phase HPLC (Clark-Lewis et al, Science 231:134-139, 1986).

Coupling is performed by mixing 8 ml of 0.1M MES (pH 4.5), 1 ml of 0.8 μm Fluoricon™ carboxyl-activated polystyrene particles 5% W/V, 1 mg of MBP or $p56^{lck}$ peptide, and 5 mg of EDC. The contents are vortexed and then incubated at room temperature overnight. The particles are sedimented by centrifugation at 6,500 rpm (6000xg) for 10 min in a Sorval 5C-5B centrifuge. The supernatant is aspirated, and the pelleted particles are washed with 20 ml of buffer A. The particles are centrifuged and resuspended in 40 m buffer A to a final concentration of 25 μg protein per ml and 0.125% W/V particles assuming 100% coupling yield. The washed particles are stored in buffer A containing 0.2% NaN_3 at 4°C. NaN_3 is removed by one washing step prior to use of the beads. Coupling of peptides or MBP via carboxy groups is performed by using Fluoricon™ 0.8 μm diameter amino activated polystyrene particles with the above coupling protocol.

EXAMPLE 2

Protein-Tyrosine Kinase Assay by PCFIA

Stock solutions of 0.1 M ATP and 1.0 M $MnCl_2$ are thawed daily. Two ml of substrate-coated particles in 20 mM Tris-HCl (pH 7.5) are mixed with 20 μl of 0.1 m ATP and 40 μl of 1.0 M $MnCl_2$ stock so that the final concentration after addition of kinase sample is 0.5 mM ATP and 10 mM $MnCl_2$. 20 μl of this substrate/particle suspension is added to each of the 96 wells of the filtration plate, either manually or automatically by the screen machine.

To test tyrosine kinase activity in sample fractions containing the protein-tyrosine kinase $p56^{lck}$, 20 μl of fractions or dilutions is transferred into the wells of the filtration plate containing assay particles, ATP and $MnCl_2$. The plates are incubated at 37°C for 15 min (maximal phosphorylation was observed after 2 min). The plates are then placed in the screen machine, and the following sequential steps are performed automatically:

the wells are drained and washed with buffer B to remove kinase; 20 µl of anti-phosphotyrosine mab PY20 or 4G10 are added at 1 µg/ml in buffer A; plates are incubated for 10 min at room temperature; wells are drained and washed with buffer B; 20 µl of polyclonal FITC conjugated goat anti-mouse antibody is added at 4 µg/ml in buffer B and incubated for 10 min. Wells are drained and washed twice in buffer B and the amount of fluorescence in each well is then determined using excitation at 485 nm and emission at 535 nm, and values are recorded as relative fluorescence units (RFU).

To compare the sensitivity of the PCFIA kinase assay to the standard [γ-^{32}P]ATP based kinase assay (filter-type assay), PCFIA conditions are modified to model conditions of the radioassay. First, buffer A is replaced with a buffer used in the [^{32}P]phosphate incorporation filter assay (buffer C), with the exception that glycerol is omitted since it clogs the 0.2 µm filter membrane in the filtration plate. Furthermore, the ATP concentration is altered to 50 µM final. Otherwise, assays are performed as the standard PCFIA kinase assay.

EXAMPLE 3

[γ-^{32}P]ATP Protein-Tyrosine Kinase Assays

Radioactive [γ-^{32}P]ATP-based kinase assays are performed as described in Sanghera et al., J. Biol. Chem. 265:52-57, 1990, with minor modifications to facilitate comparison with the PCFIA kinase assay. Under standard conditions, assays are performed in buffer C at a final volume of 25 µl. Serial dilutions of purified recombinant, p56^{lck}, are transferred into microfuge tubes to give the same final concentrations as used in the PCFIA kinase assays. To perform the assay, 15 µl of 1.65 mg/ml MBP (370 µg/ml final) is added in microfuge tubes on ice with 5 µl of p56^{lck} sample and 5 µl of 250 µM ATP containing 1 µCi of [γ-^{32}P]ATP. The mixture is incubated for 15 min at 37°C. The assay is terminated by spotting

20 µl aliquots of the reaction mixture onto 1.5 cm^2 pieces of Whatman P81 phosphocellulose paper. Substrate is allowed to absorb onto filters for 30 seconds, and non-incorporated radioactivity is removed by ten washes in 1% phosphoric acid over 2 hours. The wet filters are transferred into 6 ml plastic scintillation vials containing 2 ml of Ecolume scintillation fluid, and radioactivity is measured in a Packard scintillation counter.

In modified versions of this assay, different concentrations of MBP and specific activities of [γ-^{32}P]ATP were used as indicated herein.

EXAMPLE 4

Protein-Tyrosine Phosphatase Assay By PCFIA

MBP-coated particles are phosphorylated in vitro by mixing 10 ml of MBP-coated particles at 25 µg MBP/ml, 0.125% w/v beads in buffer A, 0.5 µM ATP, 10 µM $MnCl_2$, and approximately 70 ng of purified recombinant $p56^{lck}$. The mixture is incubated at 37°C for 30 minutes and centrifuged at 6,000 g for 10 minutes, and the supernatant is aspirated. The pellet is washed twice in 15 ml of buffer D to remove residual $p56^{lck}$ and is finally resuspended in 10 ml of buffer D (0.125% beads).

Potato acid phosphatase (PAP) is diluted in buffer D to 10 U/ml (5U/ml final in assay) and serially diluted in one in two-fold in a 96-well plate. 20 µl of PAP sample are transferred to the filtration plate wells containing 20 µl of phosphorylated MBP particles and incubated at 37°C for 15 min. The assay is then completed as described for the PCFIA kinase assay after incubation with kinase. Alkaline phosphatase (AP) assays are performed similarly, AP is diluted to 10 U/ml (5 U/ml final) and serially diluted in buffer E, and the assay is continued as above for PAP.

EXAMPLE 5

Phosphatase Inhibition

Sodium orthovanadate or sodium molybdate are diluted to 40 μM (20 μM final in assay) in buffer D containing 2 U/ml PAP. Inhibitor is serially diluted three-fold in buffer D with 2 U/ml PAP in each well of a 96-well plate. 20 μl of titrated inhibitors are transferred to filtration plate wells containing 20 μl of $p56^{lck}$-phosphorylated MBP-coated particles followed by a 15 minute incubation at 37°C. The plate is further processed as described in the PCFIA kinase method, and the fluorescence in each well is measured.

EXAMPLE 6

Protein-Tyrosine Kinase Assay by PCFIA and with MBP as a Substrate

Antiphosphotyrosine antibodies are widely used in the study of protein-tyrosine phosphorylation events. In the instant example, it is examined whether these antibodies can be exploited for the measurement of protein-tyrosine kinase or phosphatase activity. Phosphorylation or dephosphorylation of tyrosine on immobilized substrates is measured by an immunological technique using the particle concentration fluorescence immunoassay (PCFIA). The enzyme sample is directly added into wells of the filtration plate and substrate (i.e., microsphere beads coupled with suitable protein or peptide substrate). Optimal concentrations of ATP (0.5 μM) and $MnCl_2$ (10 μM) are then added. Concentrations of ATP ranging from 25 μM to 2.5 μM are found to give satisfactory results. Enzyme is removed from the substrate by an automatically performed filtration step, and the amount of phosphotyrosine residues per well are then measured using anti-phosphotyrosine mab and a fluoresceinated second antibody.

EXAMPLE 7

Sensitivity of the Assay

To assess the sensitivity of the assay, serial dilutions of partially purified preparations of the protein protein-tyrosine kinase $p56^{lck}$ from LSTRA cells are tested in a kinase assay using PCFIA, and the results are compared to results obtained in the standard filter-based assay that uses [^{32}P]-phosphate incorporation. Figure 1 illustrates the dose response curves of $p56^{lck}$ kinase activity obtained by the two methods, each performed under optimal conditions. The dose response curve obtained in the standard filter-type assay is linear, whereas the dose response of the PCFIA is non-linear. The non-linearity of the PCFIA dose response curve reflects the complexity of this phosphotyrosine detection system.

It is postulated that the non-linearity of the dose response of the PCFIA is due to the nature of the detection system that employs polyclonal fluoresceinated and anti-mouse antibodies that bind to the monoclonal anti-phosphotyrosine antibody and to the potential quenching of the fluorescent signal that increases with increasing fluorescence present in the well. Because of this non-linear dose response, unknown samples such as column fractions from biochemical purifications should be tested at several (two or three) different dilutions. This will help to better estimate the relative amounts of kinase present in the different peaks.

Using identical samples, the sensitivity of the PCFIA method is approximately 100-fold greater than the sensitivity of the standard filter-type radioassay. The PCFIA is performed using the conditions optimal for the filter-type assay and vice versa. There is about a 50% reduced signal, but a similar titration endpoint when the PCFIA is performed with the conditions used for the isotopic assay. No signal is seen in the optimized filter-type assay using PCFIA conditions (Figure 1a). Two

commercially available anti-phosphotyrosine antibodies, PY20 and 4G10, resulted in very similar signals in the assay (Figure 1b), whereas a third MAb, 1G2, is ineffective.

EXAMPLE 8

Protein-tyrosine Kinase Assay with a Synthetic Substrate

Microsphere beads coupled with a poly glutamic acid-tyrosine random copolymer (ratio 4:1) as substrate were also used in some $p56^{lck}$ assays. Although the test worked well, the signal was about two to three-fold reduced in comparison with the $p56^{lck}$ peptide coupled via the N-terminus, thus indicating that general substrates can also be used with this method.

PCFIA is also established with a synthetic peptide as substrate for the detection of $p56^{lck}$. The substrate peptide corresponds to the autophosphorylation site of $p56^{lck}$. Preliminary PCFIA experiments with a 15-amino-acid-residue peptide gives poor signals. A peptide is therefore specially designed for the PCFIA that had two N-terminal lysine residues allowing high efficiency coupling to microsphere particles. This is followed by two glycine residues that serve as spacers. The next 11 amino acids corresponded to the $p56^{lck}$ autophosphorylation site, followed by two glycine residues and a repeat of the $p56^{lck}$ autophosphorylation site. This peptide, coupled via the N-terminal lysine residues with carboxylated microsphere particles, yields identical signals to the MBP-coated beads, whereas amino-activated microsphere beads coupled with the peptide via the C-terminus has a 70% reduced signal (Figure 2). This illustrates that peptides can be used successfully in this type of assay and opens the way for use of specific peptide sequences that are recognized by individual kinases or phosphatases. The fact that the orientation in which the peptide is coupled to microsphere particles affected the signal

indicates that the assays will have to be assessed and optimized for each individual peptide substrate.

EXAMPLE 9

$p56^{lck}$ Protein-Tyrosine Kinase Activity Detection in Column Fractions Following Ion Exchange Chromatography

Protein-tyrosine kinase activity is detected in column fractions obtained after standard ion exchange chromatography using PCFIA or the standard isotope filter assay. Extracts of 10^9 LSTRA cells or 10^8 YAC cell membranes are applied to DEAE-cellulose ion exchange columns and proteins eluted by an increasing salt gradient. Figure 3a shows the protein-tyrosine kinase activity profile obtained with 1:5 dilutions as column fractions of LSTRA cell extract measured by the standard filter type assay using the synthetic $p56^{lck}$ peptide containing the site of autophosphorylation as substrate. Figure 3b shows the kinase profile obtained by testing a 1:150 dilution of the above fractions using the PCFIA and the synthetic $p56^{lck}$ autophosphorylation peptide. The profiles obtained by both methods match very well. Fractions of the YAC cell extract separated by the above chromatographic method are also tested by both kinase assays. Whereas an identical kinase elution profile was obtained with PCFIA method (Figure 3c), no [^{32}P]-incorporation could be detected with the standard filter type assay.

Application of the PCFIA technique in the analysis of cell extracts separated by ion exchange chromatography demonstrates that PCFIA compared favorably with the filter-type assay (Figure 3) with respect to sensitivity and selection. LSTRA cells overexpress $p56^{lck}$ kinase activity, and the presence of this enzyme is easily detected in the column fractions by PCFIA or the filter assay using synthetic peptide as substrate. Protein-tyrosine kinase activity in column fractions obtained from

YAC cells that do not have elevated $p56^{lck}$ levels could not be detected with the filter assay but was easily detectable with PCFIA (Figure 3c). The increased sensitivity of this assay might therefore greatly facilitates the detection and analysis of novel kinases.

EXAMPLE 10

Protein-Tyrosine Phosphatase Assay

MBP-coated particles phosphotyrosine phosphorylated by $p56^{lck}$ kinase are used as substrates for the detection of phosphatases. Serially diluted amounts of phosphatases are incubated with phosphotyrosine MBP beads and residual phosphotyrosine residues measured by PCFIA. Potato acid phosphatase at 5 U/ml catalyzes the complete removal of phosphate from phosphotyrosine residues, whereas alkaline phosphatase is not able to cleave phosphatase residues from this substrate (Figure 4a). The non-specific phosphatase inhibitors molybdate and vanadate are then tested in the PCFIA phosphatase assay by incubating serial dilutions of each of the two inhibitors with a constant amount of potato acid phosphatase and phosphotyrosine MBP beads. Both inhibitors completely inhibit the phosphatase at 20 µM concentration and have a similar dose response down to 0.2 µM (Figure 4b).

The PCFIA technique used for the detection of kinases was easily adapted to the measurements of phosphatases as shown with the example of phosphorylated MBP used as a substrate and potato acid phosphatase, an enzyme that is well known to have a very broad specificity. No activity for alkaline phosphatase, an enzyme with more restricted specificity than PAP, could be detected under the conditions used (Figure 4a). Molybdate and vanadate, both inhibitors of phosphatases, were able to block the activity of potato acid phosphatase with similar dose response curves (Figure 3b). The use of the PCFIA for the screening of potential inhibitors or

activators of kinases and phosphatases might well be one of the biggest assets of this method. Since ten plates (960 sample wells) can be screened per machine run in approximately 4 hours, the rapid screening of large numbers of substances that might specifically interact with these enzymes can be performed.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Such modifications and variations are intended to come within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ziltener, Hermann

(ii) TITLE OF INVENTION: METHOD FOR THE DETECTION OF PHOSPHOTYROSINE RESIDUES

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Seed and Berry
(B) STREET: 6300 Columbia Center, 701 Fifth Avenue
(C) CITY: Seattle
(D) STATE: Washington
(E) COUNTRY: U.S.
(F) ZIP: 98104-7092

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Hermanns, Karl R.
(B) REGISTRATION NUMBER: 33,507
(C) REFERENCE/DOCKET NUMBER: 140053.407

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-622-4900
(B) TELEFAX: 206-682-6031
(C) TELEX: 3723836

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Lys Gly Gly Arg Leu Ile Glu Asp Glu Tyr Thr Ala Arg Gln Gly
1 5 10 15

Gly Lys Arg Leu Ile Glu Asp Glu Tyr Thr Ala Arg Gln
20 25

Claims

1. A method for detecting a phosphotyrosine residue, comprising:

contacting the phosphotyrosine residue with an anti-phosphotyrosine antibody which binds to the phosphotyrosine residue, said anti-phosphotyrosine antibody labelled directly or indiretly with a fluorescent marker; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

2. A method for measuring the activity of a protein-tyrosine kinase within a sample, comprising:

immobilizing a substrate on a solid support, said substrate containing a tyrosine residues;

exposing the substrate to the sample such that the protein-tyrosine kinase within the sample phosphoralates the tyrosine residue of the substrate to form a phosphotyrosine residue;

contacting the sample with an anti-phosphotyrosine antibody which binds to the phosphotyrosine residue; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

3. A method for measuring the activity of a protein-tyrosine phosphatase within a sample, comprising:

immobilizing a substrate on a solid support, said substrate containing a phosphotyrosine residue;

exposing the substrate to the sample such that the protein-tyrosine phosphatase within the sample dephosphoralates the phosphotyrosine residue of the substrate to form a tyrosine residue;

contacting the sample with an anti-phosphotyrosine antibody which binds to the phosphotyrosine residue, said anti-phosphotyrosine antibody labelled directly or indiretly with a fluorescent marker; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

4. A method for screening a substrate for a protein-tyrosine kinase, comprising:

immobilizing the substrate on a solid support;

exposing the substrate to the protein-tyrosine kinase;

contacting the substrate with an anti-phosphotyrosine antibody, said anti-phosphotyrosine antibody labelled directly or indirectly with a fluorescent marker; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

5. A method for screening a substrate for a protein-tyrosine phosphatase, comprising:

immobilizing the substrate on a solid support;

exposing the substrate to the protein-tyrosine phosphatase;

contacting the substrate with an anti-phosphotyrosine antibody, said anti-phosphotyrosine antibody labelled directly or indirectly with a fluorescent marker; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

6. A method for screening an agent for its ability to modulate the activity of a protein-tyrosine phosphatase or its ability to modulate the specificity of a substrate to the protein-tyrosine phosphatase, comprising:

immobilizing the substrate on a solid support, said substrate containing a phosphotyrosine residue;

exposing the substrate to the protein-tyrosine phosphatase in the presence of the agent;

contacting the substrate with an anti-phosphotyrosine antibody, said anti-phosphotyrosine antibody labelled directly or indirectly with a fluorescent marker; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

7. A method for screening an agent for its ability to modulate the activity of a protein-tyrosine kinase or its ability to modulate the specificity of a substrate to the protein-tyrosine kinase, comprising:

immobilizing the substrate on a solid support, said substrate containing a kinase residue;

exposing the substrate to the protein-tyrosine kinase in the presence of the agent;

contacting the substrate with an anti-phosphotyrosine antibody, said anti-phosphotyrosine antibody labelled directly or indirectly with a fluorescent marker; and

detecting the anti-phosphotyrosine antibody by particle concentration fluorescence immunoassay.

8. The method of any one of claims 1-7 wherein the anti-phosphotyrosine antibody is indirectly labelled by contact with a fluorescently labelled antibody which binds to the antiphosphotyrosine antibody.

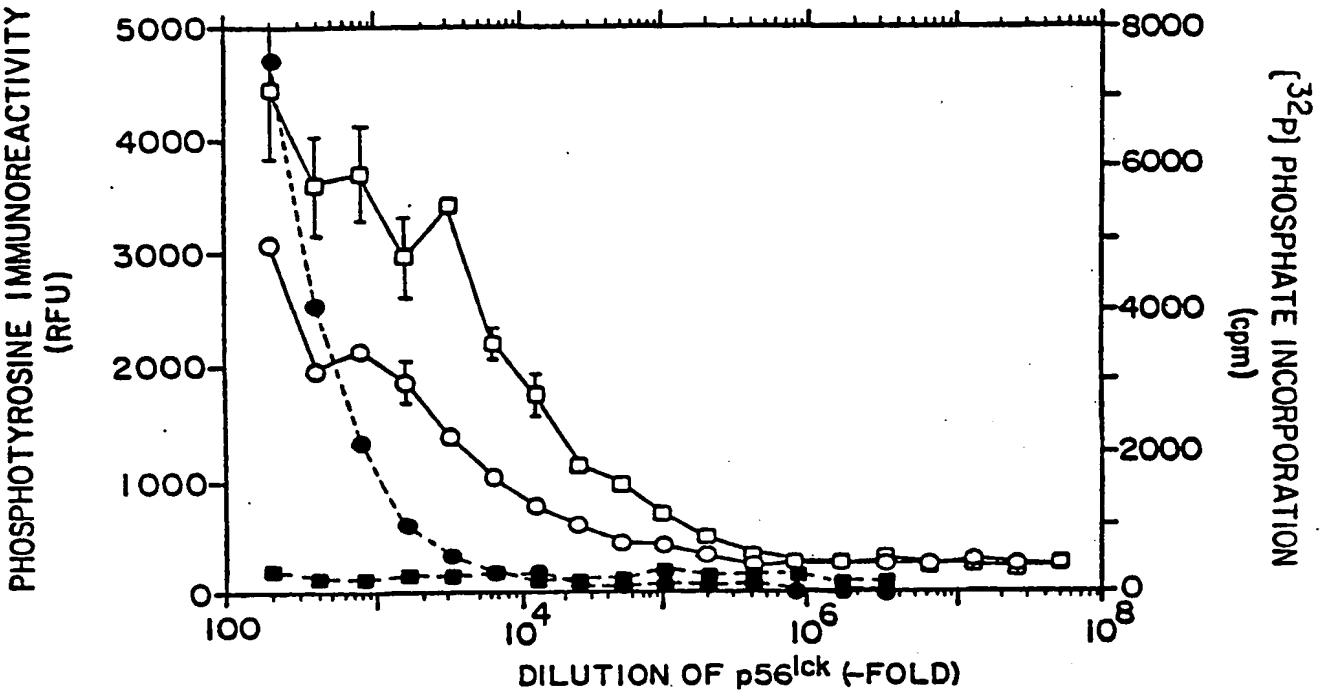
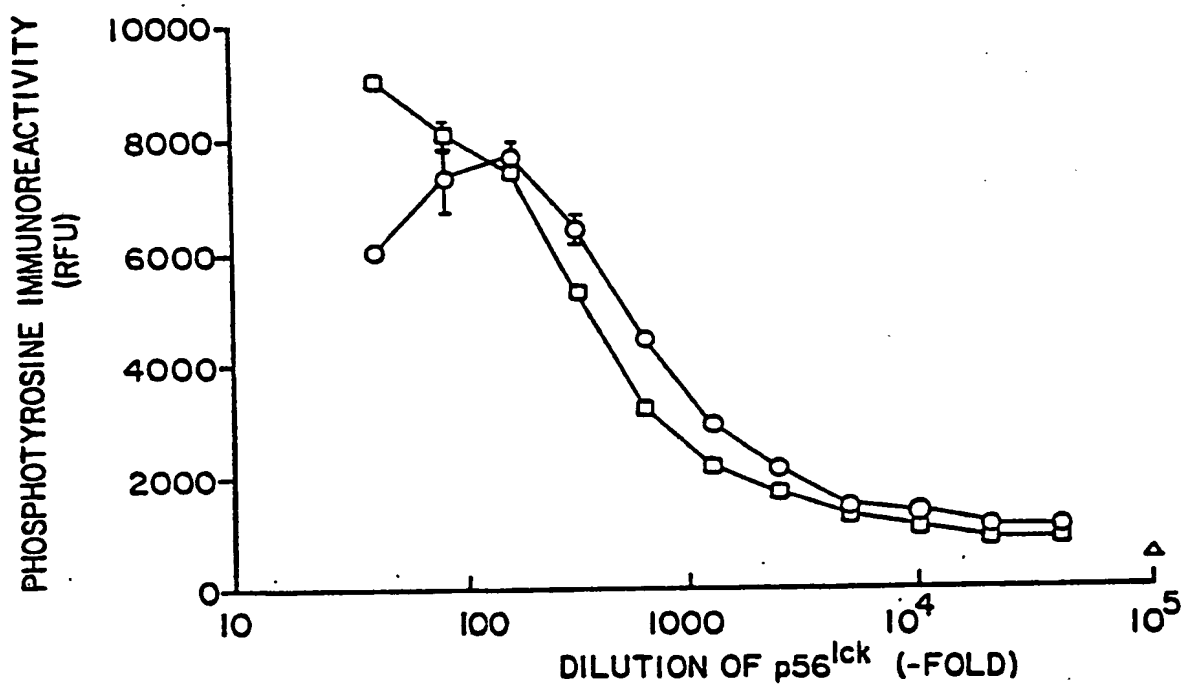

PHOSPHOTYROSINE IMMUNOREACTIVITY (RFU)
5000
4000
3000
2000
1000
0
8000
6000
4000
2000
0
[32P] PHOSPHATE INCORPORATION (cpm)
100
10^4
10^6
10^8
DILUTION OF p56lck (-FOLD)

FIG. 1A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10000
8000
6000
4000
2000
0
10
100
1000
10^4
10^5
DILUTION OF p56lck (-FOLD)

FIG. 1B

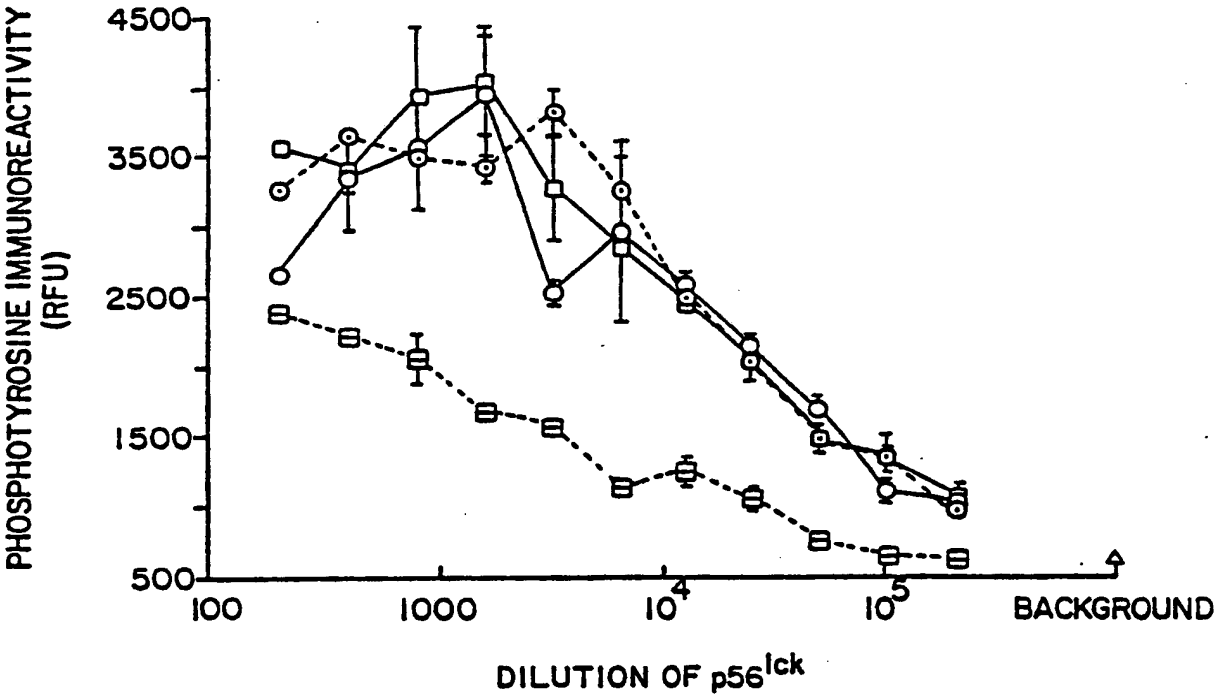
PHOSPHOTYROSINE IMMUNOREACTIVITY (RFU)
4500
3500
2500
1500
500
100
1000
10^4
10^5
BACKGROUND
DILUTION OF p56lck

FIG. 2

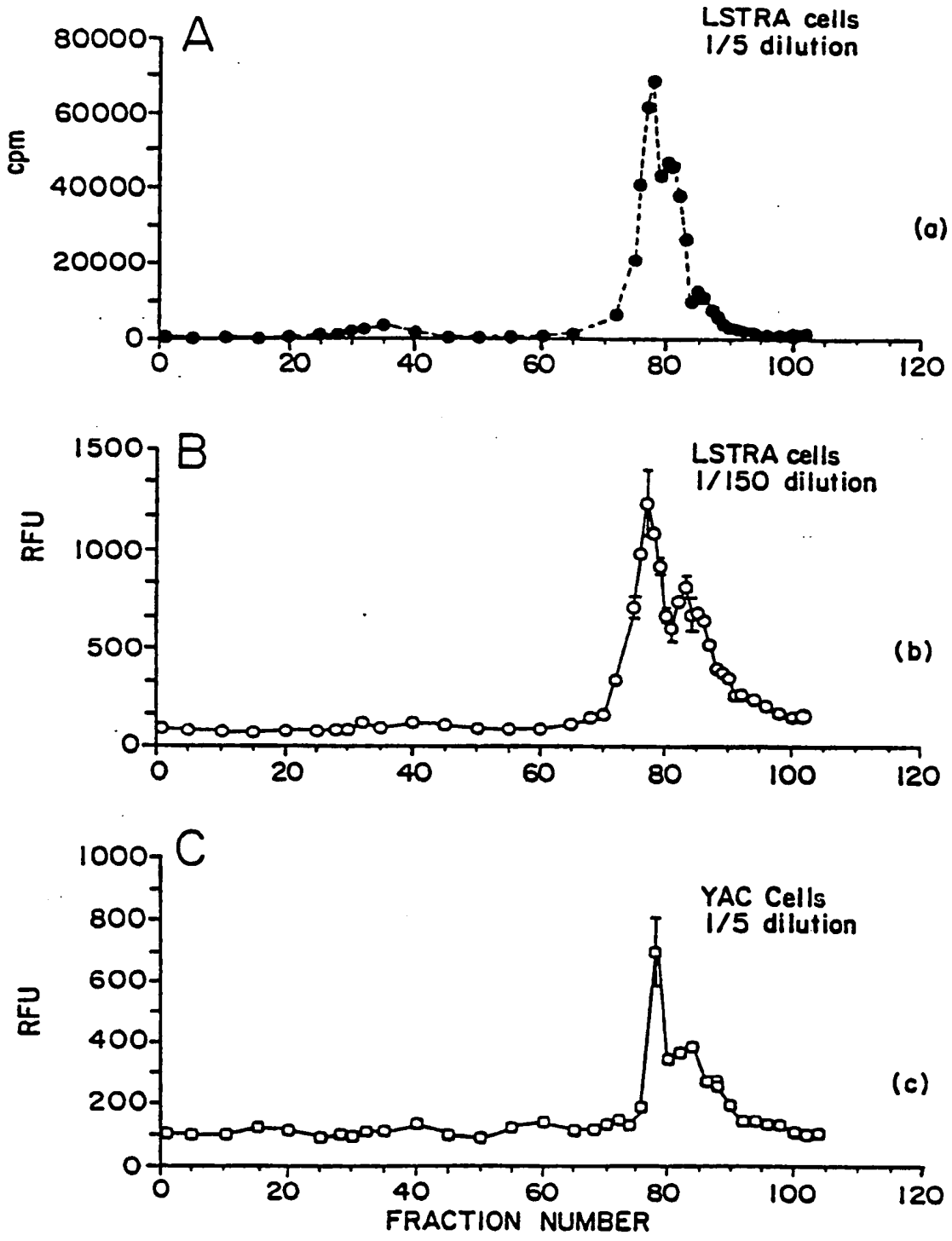
A
LSTRA cells
1/5 dilution
cpm
80000
60000
40000
20000
0
(a)
B
LSTRA cells
1/150 dilution
RFU
1500
1000
500
0
(b)
C
YAC Cells
1/5 dilution
RFU
1000
800
600
400
200
0
(c)
0
20
40
60
80
100
120
FRACTION NUMBER

FIG. 3

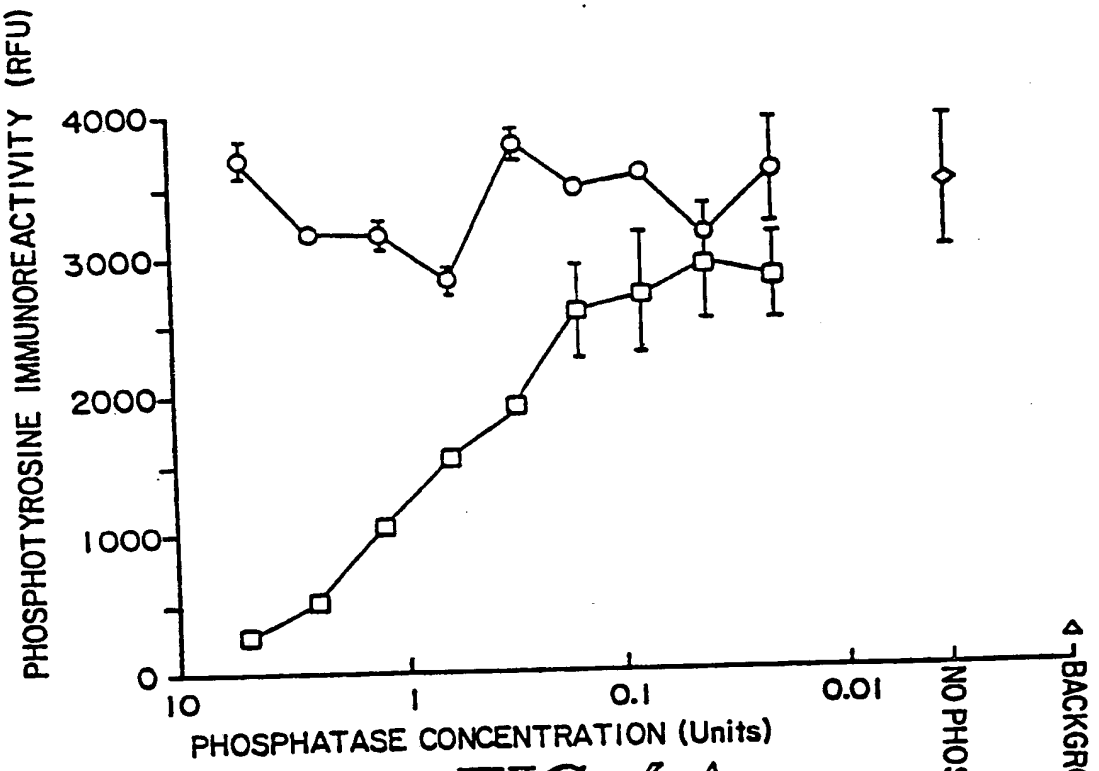

FIG. 4A

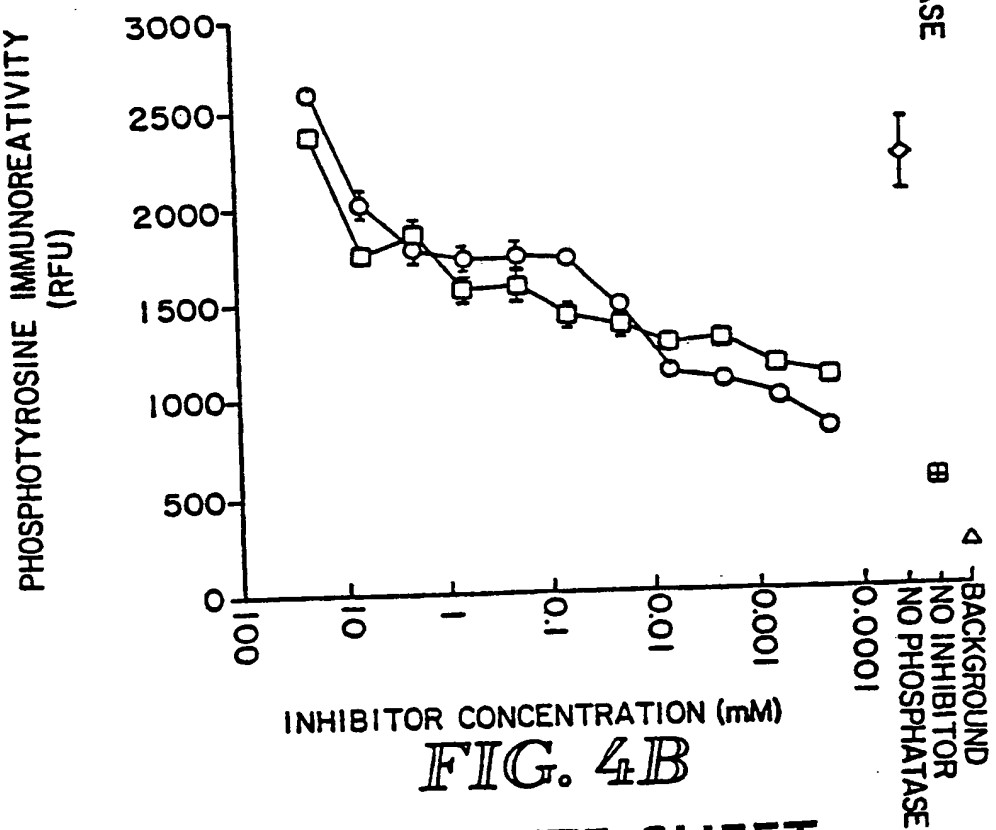

FIG. 4B

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 92/00328

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/573; G01N33/68; G01N33/546; G01N33/58
//G01N33/577

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	WO,A,9 010 234 (EURO.DIAGNOSTICS B.V.) 7 September 1990 ---	1-8
Y	CLINICAL CHEMISTRY. vol. 31, no. 9, September 1985, WINSTON US pages 1487 - 1490 CHRIS MACCRINDLE; KATHRYN SCHWENZER; MICHAEL E. JOLLEY 'Particle Concentration Fluorescence Immunoassay: A New Immunoassay Technique for Quantification of Human Immunoglobulins in Serum' see the whole document ---	1-8
P,Y	EP,A,0 449 269 (BOEHRINGER MANNHEIM GMBH) 2 October 1991	1,8
A	see the whole document ---	2-7
	-/--	

° Special categories of cited documents :[10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 OCTOBER 1992	30. 10. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	DöPFER K.P.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	US,A,453 439 (A. RAYMOND FRACKELTON,JR.; HERMAN N. EISEN; ALONZO H. ROSS) 24 September 1985 see the whole document ---	1-8
A	CHEMICAL ABSTRACTS, vol. 100, no. 19, 7 May 1984, Columbus, Ohio, US; abstract no. 154954j, JOLLEY, MICHAEL E. ET AL. 'Particle concentration fluorescence immunoassay (PCFIA): a new immunoassay technique with high sensitivity' page 401 ; see abstract & J. IMMUNOL. METHODS 67(1), 21-34 (1984) -----	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9200328 SA 62704

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/10/92
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9010234	07-09-90	NL-A-	8900529	01-10-90
		AU-A-	5263190	26-09-90
		EP-A-	0461174	18-12-91
		JP-T-	4504171	23-07-92
EP-A-0449269	02-10-91	DE-A-	4009848	02-10-91
US-A-453439		None		

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